



# Convergent evolution of marine mammals is associated with distinct substitutions in common genes

## Citation

Zhou, Xuming, Inge Seim, and Vadim N. Gladyshev. 2015. "Convergent evolution of marine mammals is associated with distinct substitutions in common genes." *Scientific Reports* 5 (1): 16550. doi:10.1038/srep16550. <http://dx.doi.org/10.1038/srep16550>.

## Published Version

doi:10.1038/srep16550

## Permanent link

<http://nrs.harvard.edu/urn-3:HUL.InstRepos:23845234>

## Terms of Use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA>

## Share Your Story

The Harvard community has made this article openly available.  
Please share how this access benefits you. [Submit a story](#).

[Accessibility](#)

# SCIENTIFIC REPORTS

OPEN

## Convergent evolution of marine mammals is associated with distinct substitutions in common genes

Received: 26 June 2015

Accepted: 15 October 2015

Published: 09 November 2015

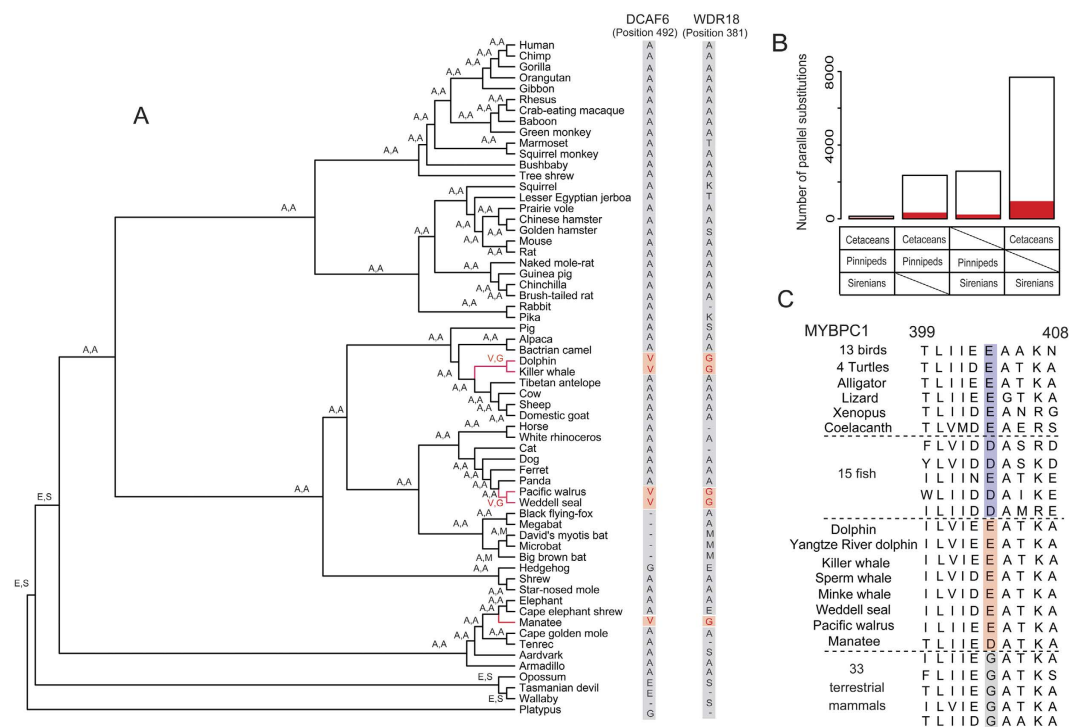
Xuming Zhou, Inge Seim & Vadim N. Gladyshev

Phenotypic convergence is thought to be driven by parallel substitutions coupled with natural selection at the sequence level. Multiple independent evolutionary transitions of mammals to an aquatic environment offer an opportunity to test this thesis. Here, whole genome alignment of coding sequences identified widespread parallel amino acid substitutions in marine mammals; however, the majority of these changes were not unique to these animals. Conversely, we report that candidate aquatic adaptation genes, identified by signatures of likelihood convergence and/or elevated ratio of nonsynonymous to synonymous nucleotide substitution rate, are characterized by very few parallel substitutions and exhibit distinct sequence changes in each group. Moreover, no significant positive correlation was found between likelihood convergence and positive selection in all three marine lineages. These results suggest that convergence in protein coding genes associated with aquatic lifestyle is mainly characterized by independent substitutions and relaxed negative selection.

It has been suggested that convergent evolution is characterized by convergent or parallel amino acid substitutions at specific amino acid sites in distant organisms<sup>1,2</sup>. For example, more than half of the substitutions (62% of 126 experimental substitutions) in two replicate populations of bacteriophage  $\phi$ X174 were parallel, and approximately 94% of candidate convergently evolved genes in a study of distant echolocating mammals (toothed whales and echolocating bats) contained parallel amino acid substitutions<sup>3,4</sup>. Given that these conclusions are typically drawn from experiments with animals and/or analyses of pairs of organisms that independently acquired a trait of interest<sup>3–6</sup> and may be subject to strong artificial selection and random substitutions, it is of interest to examine the molecular basis for convergent adaptations in three or more independent natural groups.

One such opportunity is offered by the phenotypic convergence following the transition of mammals from terrestrial to aquatic environments, which occurred at least three times independently<sup>7</sup>. Extant marine mammals include more than 120 species belonging to three distinct mammalian orders: Carnivora (walruses, sea lions, and other pinnipeds), Cetacea (whales, dolphins, and porpoises), and Sirenia (manatees and dugongs). Cetaceans and sirenians originated approximately 50 million years ago in the early Eocene, while pinnipeds trace their ancestry back to late Oligocene<sup>8</sup>. Because of similar constraints imposed by an aquatic environment, marine mammals are characterized by obvious morphological convergence, such as a streamlined body shape with modified limbs (pinnipeds have front and hind limbs modified as flippers, whereas cetaceans and sirenians completely lost their hind limbs). In addition, most marine mammals present a respiratory system adapted for reduced oxygen consumption, which enables them to withstand hypoxia and prolong deep dives<sup>8</sup>. Despite a rich history of the field, specifically

Division of Genetics, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA. Correspondence and requests for materials should be addressed to V.N.G. (email: vgladyshev@rics.bwh.harvard.edu)



**Figure 1. Parallel and unique substitutions in marine mammals.** (A) Parallel substitutions in DCAF6 and WDR18 in marine mammals. We utilized genomic data of 5 marine mammals and 57 terrestrial mammals with completed genomes. Amino acid residues associated with each branch are based on reconstructed ancestor sequences at the corresponding positions in DCAF6 and WDR18. (B) Number of parallel substitutions along the branches of the three marine groups, or at least two marine mammal lineages, since they evolved from a terrestrial ancestor. Parallel substitutions unique to the indicated marine groups are shaded red. (C) Deduced partial amino acid sequence alignment of MYBPC1. The common substitution identified is located at amino acid position 404 of the human ortholog. The corresponding sites in terrestrial mammals are shown in gray, in marine mammals in orange, and in fish, birds, amphibians, and reptiles in blue.

in the context of physiological adaptations of marine mammals, relatively little is known about the molecular changes that underlie convergent genetic adaptations associated with aquatic life. Several previous studies describing mutations in marine mammals have focused on cetaceans. For instance, Wang *et al.*<sup>9</sup> reported that *HOXD12* is under Darwinian selection and associated with two episodes of cetacean forelimb reorganization. Mirceta *et al.*<sup>10</sup> reported an adaptive molecular signature where elevated myoglobin (MB) net surface charge in cetaceans and pinnipeds is mechanistically linked with maximal myoglobin concentration. Very recently, Foote and colleagues<sup>11</sup> performed genome sequencing of three species of marine mammals (the killer whale, walrus, and manatee) and interpreted the convergent evolution of marine mammals by counting parallel amino acid substitutions in their genomes. No apparent correlation between functional enrichment of genes with parallel substitutions and aquatic adaptation was found, with the observed parallel substitutions also detected throughout genomes of terrestrial taxa with no obvious phenotypic convergence<sup>11</sup>. Here, aided by whole genome alignments of a greater number of mammalian species and evolutionary model analyses, we reexamined the contribution of parallel substitutions to genomic convergence in the three distinct marine lineages and identified a subset of genes which may contribute to the aquatic phenotype.

## Results

**Most parallel substitutions are not unique to marine mammals.** To investigate molecular convergence, we first identified parallel amino acid residue changes in the three groups of marine mammals. A parallel substitution was defined as an amino acid change at the same position in marine mammals different from that of the ancestral node of each marine group, but identical in the three marine groups. Aided by human UCSC whole-genome multiple alignments, protein-coding sequences representing 5 marine and 57 terrestrial mammals were obtained, and ancestral sequences of each node in the phylogenetic tree were reconstructed (Fig. 1A, Supplementary Fig. S1). In total, 133 parallel amino acid residue substitutions in 132 genes were identified in all three marine groups, as well as 2,351, 7,684, and 2,579

parallel substitutions in cetaceans + pinnipeds, cetaceans + sirenians, and pinnipeds + sirenians, respectively (Fig. 1B, Supplementary Table S1).

Independent of the analysis of parallel substitutions, we considered all mammalian species in the data set and identified unique substitutions in marine mammals, which were defined as any amino acid residue at the same position in all three marine mammal groups that is neither found in the ancestral nodes with their respective terrestrial taxa nor in other terrestrial mammals. This scan identified 26 genes with unique amino acid changes in 8 marine mammals (Supplementary Table S2). Some of the unique changes in marine mammals are of interest because the genes are associated with phenotypic changes or physiological functions indicative of aquatic adaptation. For example, *MYBPC1* encodes a slow skeletal muscle isoform of myosin-binding protein C that supports muscle contraction by recruiting muscle-type creatine kinase to myosin filaments<sup>12,13</sup>. *KCNQ4* encodes Kv6.3, which complexes with oxygen-sensitive Kv2 potassium channel subunits to regulate vascular tone<sup>14</sup>. *CPT2*-encoded protein controls fatty acid oxidation<sup>15</sup>, and mutations in *TMC8* are associated with an autosomal recessive genetic hereditary skin disorder in humans<sup>16</sup>.

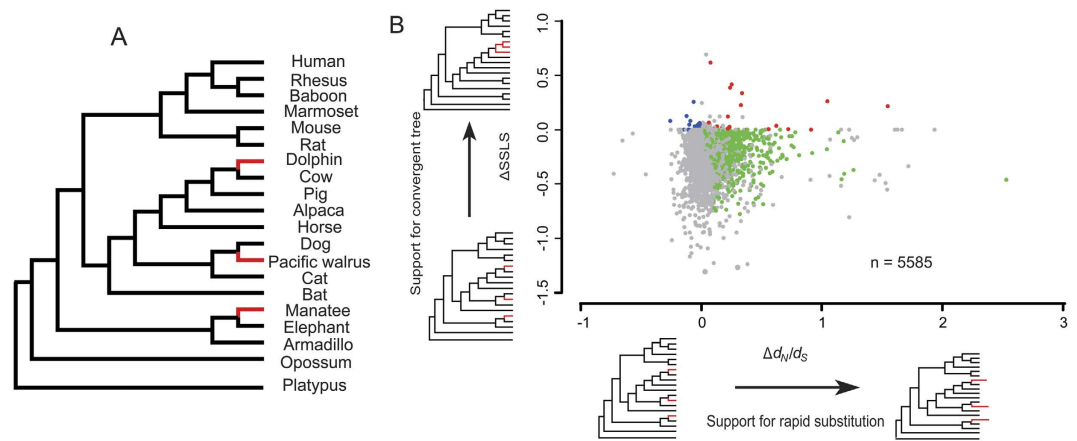
If parallel substitutions represent a measure of genomic convergence, one may ask how many parallel substitutions are unique to groups of interest and whether the number of such substitutions exceeds that of control groups. Based on the parallel substitutions and unique amino acid changes identified in the three marine lineages, we found that the majority of substitutions were not unique to marine groups (i.e., they are also present in some terrestrial mammals) (Fig. 1B). In particular, of the 133 parallel substitutions in the three marine groups, only two (substitutions in *DCAF6* and *WDR18*) were unique to all marine mammals (Fig. 1A). For parallel substitutions in two marine groups, 10.5%, 11.4%, and 8.3% represented unique changes in cetaceans + pinnipeds, cetaceans + sirenians, and pinnipeds + sirenians, respectively (Fig. 1B). Thus, the majority of parallel substitutions in marine mammals also occur in terrestrial mammals. In addition, when we replaced one of the marine lineages with its terrestrial sister taxa, parallel substitutions in the three marine lineages still did not exceed the numbers in any two marine groups plus the control terrestrial taxa (Supplementary Fig. S2). Nevertheless, *DCAF6* and *WDR18* represent candidate genes for further functional investigation. *DCAF6* functions as a ligand-dependent coactivator of nuclear receptors and interacts directly with androgen receptor (AR)<sup>17</sup>. The regulation of male skeletal integrity by AR signaling can be attributed to both osteoblasts and osteocytes<sup>18</sup>. *WDR18* encodes a WD-repeat protein that is highly conserved among vertebrate species and prior investigations have shown that *WDR18* has an essential role in determination and regulation of zebrafish laterality<sup>19</sup>.

**Backward substitutions in marine mammals.** Interestingly, the common amino acid residue change observed in marine mammalian *MYBPC1* (Gly404Glu/Asp) is also found in fish, amphibians, reptiles, and birds (Fig. 1C, Supplementary Fig. S3). Since the function of the Ig domain of myosin-binding protein C, in which this residue is located (Supplementary Fig. S4), is not known, the specific role of this 'backward' substitution in marine mammals is unclear. Interestingly, a recent study found that mutations in *MYBPC1* are associated with distal arthrogryposis type 1, a disorder characterized by joint deformities that restrict movement in the hands and feet of humans<sup>20</sup>. In addition, the corresponding malgenic mutations result in a bent body curvature and decreased motor activity in zebrafish<sup>21</sup>. Thus, we hypothesize that parallel substitutions in *MYBPC1* may be associated with limb development and skeletal muscle function in marine mammals.

The site altered in *CPT2* of marine mammals (Arg/His37Gln/Lys) is also changed in fish (Arg37Lys/Ser) and the semi-aquatic platypus (Arg37Lys) (Supplementary Fig. S3). Carnitine palmitoyltransferase II (*CPT2*) plays important roles in fatty acid oxidation and energy metabolism. Mutations in *CPT2* are associated with carnitine palmitoyltransferase II deficiency<sup>22</sup>, disabling the use of certain lipids for energy generation, particularly during fasting. A recent study found that *CPT2* gene expression is significantly increased in the liver of rabbits fed Omega-3 fatty acids<sup>23</sup>. Given the similar nutrient sources of marine mammals, platypus, and some fish, we surmise that the parallel substitution in *CPT2* may correlate with dietary preferences in the aquatic environment.

**Identification of genes associated with aquatic adaptation by evolutionary model analyses.** Since the majority of parallel substitutions are not unique to marine mammals and it is generally thought that orthologs in mammals are functionally conserved, it is doubtful that the large number of non-unique parallel substitutions and/or rare unique amino acid changes significantly contributes to phenotypic and physiological convergence in marine mammals. Thus, alternative methods are necessary. To identify genes that may be associated with an aquatic lifestyle, we next considered evolutionary models and examined whether candidate genes are enriched with parallel substitutions.

Taking into account completeness of available genomes and the number of species feasible for the scale of such analyses, the phylogenomic data consisted of 5,585 protein-coding orthologs from 20 mammals, representing all four major mammalian clades (Fig. 2A). We selected three marine mammals (the common bottlenose dolphin, walrus, and manatee; which represent cetaceans, pinnipeds, and sirenians, respectively) to ensure that the elevated substitution rate and sitewise log-likelihood support measured in the evolutionary analyses were due to inter-order substitutions rather than substitutions within each marine group.

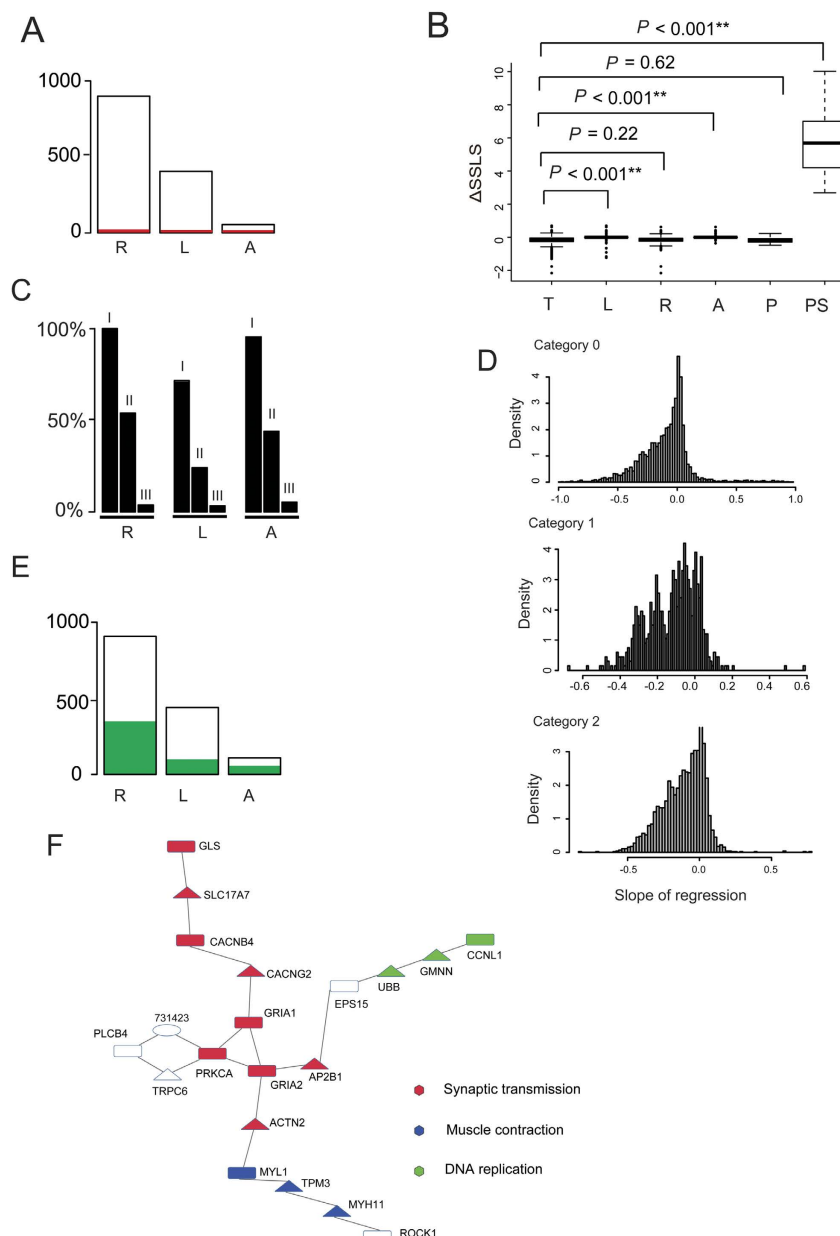


**Figure 2. Evolutionary model analyses of genomic convergence of marine mammals.** (A) Phylogenetic relationship of mammals used to identify rapidly and convergently evolved genes. Branches representing independent evolution of marine mammal lineages, for which tests for rapid substitution, likelihood convergence, and positive selection were performed, are colored red. Branches of the terrestrial taxa control set, are cow, dog, and elephant, respectively. (B) Distribution of  $\Delta d_N/d_S$  and  $\Delta SSLS$  in 5,585 orthologs (n). Loci identified by  $\Delta SSLS$ ,  $\Delta d_N/d_S$  and both methods are shown in blue, green and red, respectively, and  $\Delta SSLS$  shown here is estimated from an alternative topology ( $H_A$ , tree 1) as opposed to a common species tree ( $H_0$ ) (indicated by red branches to the left and below the plot).

We first examined genes with accelerated rates of amino acid substitutions in aquatic mammals (measured by the ratio of nonsynonymous to synonymous substitutions,  $d_N/d_S$ ) using the branch model<sup>24</sup>. For each gene, this model assigns a  $d_N/d_S$  value to a ‘foreground branch’ (marine mammals) and another  $d_N/d_S$  value to a ‘background branch’ (terrestrial mammals). The branch model (non-neutral) is compared to a null model (neutral model with single estimated  $d_N/d_S$ ), an LRT (likelihood ratio test) applied to gauge the likelihood of the evolutionary models. Genes with significantly positive  $\Delta d_N/d_S$  ( $d_N/d_S$  being larger in marine mammals than in terrestrial mammals) were defined as rapidly evolving genes in marine mammals. This analysis yielded 907 genes (16.2%, LRT  $P \leq 0.05$ ; 142 genes after multiple testing adjustment) with significantly elevated protein sequence substitution rate in marine lineages compared to terrestrial mammals (Fig. 2B, Supplementary Figs S5 and S6).

To identify sequence convergence between marine mammal lineages, we employed a maximum likelihood (ML) approach<sup>4,25</sup>, hereafter termed likelihood convergence analysis. In this method, we examined each amino acid along a given gene alignment (CDS) and measured its fit (sitewise log-likelihood support; SSLs) to the commonly accepted species tree (termed  $H_0$ ) and to nine alternative topologies in which marine taxa were ‘forced’ into erroneous monophyletic clades (representing the convergence hypothesis; see Methods). The likelihood convergence analysis revealed 441 genes (7.9%, indexed by  $\Delta SSLS$  and denoted likelihood convergence genes; 116 genes if  $\Delta SSLS$  from all nine alternative hypotheses supported the convergence) with significant likelihood support for the grouping of marine mammals as opposed to the distribution based on the canonical species tree (Supplementary Figs S7–S9).

**Aquatic adaptation genes are characterized by neither parallel substitutions nor congruent positive selection.** Having identified putative convergent genes in marine mammals by evolutionary model analysis, we assessed the prevalence of parallel substitutions in the 20-mammal dataset. Using the commonly accepted ‘species tree’ shown in Fig. 2A<sup>26–29</sup> and a likelihood method based on ancestral sequence reconstruction<sup>30</sup>, we identified 44 parallel substitutions in 38 genes in all marine mammals. As revealed by the aforementioned results for 62 mammals, the majority of parallel substitutions were not unique to each combination of two or three marine mammals (Supplementary Fig. S10). Similarity, only 14 rapidly evolved genes (1.5%) contained parallel substitutions (2.8% after multiple testing adjustment) and 6 likelihood convergence genes (1.4%) contained parallel substitutions in marine mammals (Fig. 3A). Of the 116 likelihood convergence genes (supported by  $\Delta SSLS$  from 9 alternative hypotheses), only 3 genes contained parallel substitutions in marine mammals. We further examined the relative abundance of parallel substitutions in 70 genes (hereafter denoted as candidate aquatic adaptation genes), which received evidence from both elevated substitutions rate and likelihood scores support (with  $\Delta d_N/d_S > 0$ , LRT  $P \leq 0.05$  and  $\Delta SSLS > 0$ ,  $P \leq 0.05$ ) (Supplementary Table S3); only 5.7% of the 70 genes had parallel substitutions (Fig. 3A). It is worth noting that  $\Delta SSLS$  values of parallel substitutions were significantly greater than the average  $\Delta SSLS$  of all orthologs ( $P \leq 0.01$ , Student’s t-test), suggesting that most of the parallel substitutions supported the convergence hypothesis rather than species tree. However, there was no significant difference in average  $\Delta SSLS$  between genes containing parallel substitutions and total orthologs ( $P \geq 0.05$ , Student’s t-test) (Fig. 3B).



**Figure 3. Statistics of rapidly evolving genes and likelihood convergence genes, and their functional enrichment.** (A) Percentage of genes with parallel substitutions in rapidly evolving genes (R), likelihood convergence genes (L) and 70 aquatic adaptation genes (A). Red region denotes the proportion of parallel substitutions in marine mammals. (B) Statistics of average  $\Delta SLS$  of all orthologs (T), rapidly evolving genes (R), likelihood convergence genes (L), 70 aquatic adaptation genes (A), genes containing parallel substitutions (P), and parallel substitution sites (PS). (C) Percentage of genes with unique amino acid changes in rapidly evolving genes (R), likelihood convergence genes (L) and 70 aquatic adaptation genes (A). Unique amino acid changes were classified into those changing in all three lineages of marine mammals (III), in any two of the three marine lineages (II) and in single lineages of marine mammals (I). (D) The coefficient (slope) for locus-wise regressions between sitewise support for convergence and sitewise  $d_N/d_S$  for sites under purifying selection (Category 0), neutral site (Category 1), and sites under diversifying selection (Category 2) are plotted. In each plot, there are loci showing negative relationship, characterized by slopes significantly below zero, and loci showing positive relationship, with slopes greater than zero. (E) Percentage of positively selected genes in rapidly evolving genes (R), likelihood convergence genes (L) and 70 aquatic adaptation genes (A). Positively selected genes were identified in cetaceans, pinnipeds, and sirenians, respectively. (F) Enriched network of 70 aquatic adaptation genes. Candidate genes are indicated by grey filled squares, missing genes by white filled triangles, and metabolites by white filled circles. Gene ontology categories are represented by Synaptic transmission in red, DNA replication in green, and Muscle contraction in blue.



We next searched for unique amino acid residue changes in all three marine groups, any two marine groups and single marine groups. Less than half of the rapidly evolving genes and likelihood convergence genes had unique changes in all three or any two marine groups (with less than 10% in all three marine groups). Strikingly, most of the genes (99.7% of rapidly evolving genes and 69.2% of likelihood convergence genes) contained distinct changes in each group of marine mammals (Fig. 3C). A similar trend was observed for the 70 candidate aquatic adaptation genes (Fig. 3C, Supplementary Table S4). These data show that candidate genes for convergent evolution of aquatic mammals are primarily characterized by distinct amino acid changes in each marine group.

Previous studies suggested that sequence convergence is a consequence of natural selection<sup>4</sup>. Of the 907 rapidly evolving genes in marine mammals, only 13 genes (1.4%) had a nonsynonymous to synonymous substitution ( $d_N/d_S$ ) ratio greater than one, indicating that the elevated nonsynonymous to synonymous substitutions rate in marine mammals reflects reduced purifying selection rather than positive selection (Supplementary Fig. S6). We further investigated whether the elevated  $d_N/d_S$  in marine mammals was caused by the sitewise log-likelihood support ( $\Delta$ SSLs). For each ortholog, we fitted the linear relationship between  $\Delta$ SSLs and the corresponding sitewise  $d_N/d_S$  in marine lineages and classified sites into three categories, i.e. evolved under purifying-, neutral-, or diversifying- (positive) selection in marine mammals (Fig. 3D). This effort revealed that the majority of genes presented a negative correlation between sitewise  $d_N/d_S$  and support for convergence (67.2%, 80.0%, and 75.4% of genes under purifying selection, neutrally, and diversifying selection, respectively). In particular, of 982 genes under diversifying selection with significant correlation between sitewise  $\Delta$ SSLs and sitewise  $d_N/d_S$ , only 22 genes (2.2%) showed positive correlation, indicating that sitewise convergence in marine mammals is not achieved by positive selection.

We further considered individual marine lineages. Positively selected genes (PSGs) in single marine lineage, i.e., cetaceans, pinnipeds, and sirenians, were determined using the branch site model<sup>31</sup>, respectively. Rapidly evolving genes contained a total of 350 PSGs (38.6%), likelihood convergence genes contained 49 PSGs (11.1%) and, in addition, half (50%, 35 genes) of the 70 candidate aquatic adaptation genes were PSGs (Fig. 3E). These data suggest that candidate aquatic adaptation genes (rapidly evolving genes or likelihood convergence genes) are co-influenced by lineage-specific selection with amino acid changes at distinct sites, a phenomenon previously observed for sodium channels associated with convergent evolution of electric organs in fish<sup>32</sup>.

**Functions of candidate aquatic adaptation genes.** The specific roles of the candidate genes associated with aquatic adaptation require future functional analyses. However, insights can be gained by functional gene ontology (GO) enrichment analysis. Analysis of the 70 candidate genes with elevated protein sequence substitution rates and likelihood support revealed enrichment of GO terms such as synaptic transmission (GO:0007268,  $P = 1.90 \times 10^{-4}$ , Fisher's exact test) and transmission of nerve impulse (GO:0019226,  $P = 5.10 \times 10^{-4}$ , Fisher's exact test) (*PRKCA*, *GRIA2*, *GRIA1*, *LIN7C*, *CACNB4*, *NOVA1*, *PARK7*) (Supplementary Table S5). The corresponding KEGG pathways over-represented among the candidate aquatic adaptation genes were long-term depression ( $P = 3.70 \times 10^{-4}$ , Fisher's exact test) and long-term potentiation ( $P = 3.50 \times 10^{-4}$ , Fisher's exact test) (Supplementary Table S6). In agreement, pathway analyses revealed a cellular network comprising 20 genes, including 10 of our candidate genes, centered on synaptic transmission and muscle contraction (Fig. 3F). Nerve activity is a major control mechanism of the muscle fiber type profile, and multiple signaling pathways have been implicated in activity-dependent changes of muscle fibers<sup>33</sup>. For example, *MYL1* encodes fast skeletal muscle myosin alkali light chains<sup>34</sup>. Four genes (*PRKCA*, *PLCB4*, *ROCK1*, *MAPK10*) in the Wnt signaling pathway were also present in the candidate aquatic adaptation gene list ( $P = 0.007$ , Fisher's exact test) (Supplementary Table S6). Altered expression of genes associated with the Wnt pathway has been implicated in metabolic and structural transformation of Weddell seal skeletal muscle from a strictly terrestrial lifestyle as a pup to an aquatic lifestyle adapted for deep dives as adult animals<sup>35</sup>.

Candidate aquatic adaptation genes were also significantly enriched for the terms mRNA stabilization (GO:0048255,  $P = 2.80 \times 10^{-4}$ , Fisher's exact test) and RNA stabilization (GO:0043489,  $P = 2.80 \times 10^{-4}$ , Fisher's exact test) (*HNRNPD*, *PABPC1*, *YBX1*). In response to hypoxic challenge a series of cellular responses are initiated in mammalian cells, triggering adaptive processes including changes in cell division, survival, motility, or differentiation<sup>36</sup>. Hypoxia markedly decreases total *de novo* transcription and, accordingly, several RNA-binding proteins (RBPs) have been found to affect the stability of many specific mRNAs. For example, accelerated mRNA decay is observed in response to hypoxia, and this process involves decay-promoting RBPs such as *HNRNPD* and *PABPC1*<sup>37</sup>. The mRNA-protein complexes transition into stress granules, highly specialized cytoplasmic structures and sites of mRNA storage that facilitate translational reprogramming<sup>38</sup>. A recent study demonstrated a role for *YBX1* in stress granule formation and tumor progression<sup>39</sup>. Taken together, the candidate aquatic adaptation genes encompass a plausible range of functional categories associated with marine lifestyles.

## Discussion

Our whole genome analyses revealed that parallel substitutions are widespread in marine mammals, consistent with a recent study by Foote and colleagues<sup>11</sup>. However, the majority of parallel substitutions are not unique to marine mammals. These findings do not rule out that parallel substitutions contribute

to functional and phenotypic convergence of marine mammals. As the number of taxa increases, the likelihood of finding another homoplasious substitution in a terrestrial taxon increases as well. It should be appreciated that it is currently difficult to examine whether such signatures represent an unrelated homoplasy (if molecular convergence really has occurred amongst focal taxa), or a 'true negative' (all homoplasies have occurred neutrally and there is no molecular convergence). Here, by considering different classes of substitutions and sitewise  $\Delta$ SSLS, we propose that rapidly evolving genes and sequence convergence in marine mammals are predominantly characterized by independent rather than parallel substitutions.

Two lines of evidence from experimental studies support this hypothesis. For example, the transfer of three related bacteriophage species to a novel environment revealed a high rate of parallel genetic evolution at orthologous nucleotide and amino acid residues within, but not between, species<sup>40</sup>. Similarly, a study involving *Pseudomonas aeruginosa* demonstrated that antibiotic resistance and pathogen fitness in individual isolates stemmed from parallel substitutions in four antibiotic resistance genes coupled with distinct substitutions in more than hundred other genes<sup>41,42</sup>. Following submission of our manuscript, two studies were published arguing that there is no excess of (parallel) convergence between echolocating mammals<sup>43,44</sup>. They demonstrated that methods solely based on sitewise likelihood convergence are not adequate for measuring genomic convergence<sup>44</sup>. This is consistent with our data and hypothesis, as non-unique parallel substitutions could be generated by chance in any pair of mammals following phylogenetic distance. Therefore, a combination of different evolutionary models, for example, the use of substitution rate and likelihood scores, as employed in the present study, may be necessary for future studies of convergent evolution.

It should be noted that multiple factors, including genome size and complexity, may influence whether parallel mutations occur at the same nucleotide positions or in the same gene (or complex locus)<sup>45</sup>. In the case of marine mammals, there may be two additional factors that result in distinct substitutions in common genes. First, terrestrial mammals returned to the sea at different evolutionary periods and, thus, under different genomic and environmental contexts. Second, it is clear that the adaptation to aquatic environments required multiple episodes of phenotypic convergence and likely involved multiple potentially adaptive mutations. Third, convergence generally involves four types of molecular evolution<sup>46</sup>. Although sequence convergence is the easiest to detect, other mechanisms (functional, mechanistic and structural convergence) may also play critical roles in the convergent evolution of marine mammals. Two types of mutation changes which may correlate with the aquatic adaptation of marine mammals have not been interrogated in the present study: mutations in distinct genes associated with the same pathway and mutations in *cis* regulatory regions. For example, distantly-related electric fish show both sequence convergence<sup>32</sup> and mechanistic convergence in the form of common gene expression patterns enabling the development of electric organs<sup>47</sup>. In addition, regulatory mutations in *PITX1* show signatures of positive selection in pelvic-reduced populations<sup>48</sup>. Future studies, for example functional or genomic analyses of non-coding regions, of marine mammals may complement our present study and provide further insights into the genomic basis of aquatic adaptations in mammals.

## Methods

**Data collection.** Coding sequences (CDS) of 62 mammals were obtained from the human UCSC 100 species whole-genome multiple alignment (build 04.15.2014)<sup>49</sup>. For genes with many reference sequences, the longest coding sequence was chosen. To confirm common changes in cetaceans that had not been included in the human UCSC 100 species, coding sequences of the Yangtze River dolphin (*Lipotes vexillifer*, assembly accession: GCA000442215.1), common minke whale (*Balaenoptera acutorostrata*, assembly accession: GCA000493695.1), and sperm whale (*Physeter catodon*, assembly accession: GCA000472045.1) were retrieved from NCBI by parsing genomic annotation files. In order to conduct evolutionary model analyses, coding sequences from species shown in Fig. 2A were interrogated. Cow (*Bos taurus*, UMD3.1), dog (*Canis lupus familiaris*, CanFam3.1), nine-banded armadillo (*Dasypus novemcinctus*, Dasnov3.0), human (*Homo sapiens*, GRCh38), rhesus (*Macaca mulatta*, MMUL 1.0), baboon (*Papio anubis*, PapAnu2.0), marmoset (*Callithrix jacchus*, C\_jacchus3.2.1), African elephant (*Loxodonta africana*, Loxafr3.0), mouse (*Mus musculus*, GRCm38.p3), (bottlenose) dolphin (*Tursiops truncatus*, turTru1), pig (*Sus scrofa*, Sscrofa10.2), alpaca (*Vicugna pacos*, vicPac1), horse (*Equus caballus*, EquCab2), cat (*Felis catus*, Felis\_catus\_6.2), large flying fox (*Pteropus vampyrus*, pteVam1), platypus (*Ornithorhynchus anatinus*, OANA5) and gray short-tailed opossum (*Monodelphis domestica*, monDom5) were downloaded from Ensembl (Release 77). (Florida) manatee (*Trichechus manatus latirostris*) (Assembly accession: GCA000243295.1) and (Pacific) walrus (*Odobenus rosmarus divergens*) (Assembly accession: GCA000321225.1) sequences were obtained from NCBI Genomes.

**Identification of parallel amino acid substitutions.** To identify parallel amino acid changes in marine mammals, the phylogenetic tree of 62 mammals used for reconstruction of ancestral sequences was trimmed from the original tree, with branch length from the human UCSC 100 species whole-genome multiple alignment. The ancestral sequences for each node were reconstructed with the help of FastML 3.1, which employs maximum likelihood algorithms and an empirical Bayesian approach taking into account the rate variation among sites<sup>30</sup>. We allowed FastML 3.1 to estimate the branch length of the



phylogenetic tree for each gene when the ancestral sequences were reconstructed using the set of 20 mammals. The orthologous relationship of 51 genes in three other mammals was determined by best hit in local BLAST<sup>50</sup>, and protein-coding gene sequences were aligned with MUSCLE<sup>51</sup>. Amino acid changes of interest were manually validated.

**Evolutionary model analysis pipeline.** In the evolutionary analyses, coding sequences (CDS) of individual genes from the human genome were used to query the other 19 genomes (listed in the Data collection section above and shown in Fig. 2A) for homologous sequences using reciprocal BLAST. Prior to using human genes to query genomes, we discarded sequences in which the CDS contained less than 150 nucleotides and CDS length was not divisible by 3. For genes with alternatively spliced transcripts in the human genome, the CDS with the greatest number of nucleotides were used in further analyses. Only hits with a BLAST e-score less than  $1 \times 10^{-5}$  were considered orthologs, and for cases in which there were multiple BLAST hits, the longest hit was chosen. Orthologous protein-coding genes from 20 mammals were aligned according to their translated amino acids using TranslatorX<sup>52</sup> and MUSCLE<sup>51</sup>. PAL2NAL<sup>53</sup> was used to identify conserved blocks for subsequent analyses. All analyses were run on the Orchestra supercomputing cluster supported by the Harvard Medical School Research Information Technology Group.

**Identification of rapidly evolving genes.** To identify heterogeneous rates of protein evolution in marine mammals and terrestrial mammals, the branch model<sup>24</sup> in the program *codeml*, available in the PAML package<sup>54</sup>, was used to fit each alignment and estimate differences in  $d_N/d_S$  within the tree. The branch site model<sup>31</sup> was used to detect positively selected genes in each marine lineage, respectively. A false discovery rate (FDR, Bonferroni method) correction was applied to account for multiple hypothesis testing. At least three rounds of analyses were performed to make sure that rapidly evolving genes were not affected by different start  $d_N/d_S$  values (from 0.8 to 1.5).

**Detection of likelihood convergence genes.** To detect signatures of molecular convergence, we fitted the orthologous CDS alignment data to a null model ( $H_0$ , species tree) and an alternative model ( $H_A$ , the constraint of monophyly of marine mammals). Next, we used the mean  $\Delta$ SSLs (sitewise log-likelihood support) of all sites in a gene to index the strength of support for convergence<sup>4</sup>. This method directly compares the goodness-of-fit of each gene to a pair of phylogenetic trees under a given model. SSLs for each gene alignment was estimated by RAxML v7.4.8<sup>55</sup>, and  $\Delta$ SSLs in the present study was calculated as  $\Delta$ SSLs =  $\ln L(H_A) - \ln L(H_0)$ . For example, genes with a better model fit to  $H_A$  (supporting convergence) will have positive  $\Delta$ SSLs scores, whereas genes with a better fit to species tree ( $H_0$ ) will have negative  $\Delta$ SSLs scores. We further estimated significance of convergence by performing analyses on simulated datasets. Fifty genes were randomly chosen to represent the alignment length and heterogeneity of our data. The simulations were carried out using Seq-Gen 1.3.3<sup>56</sup>, with 50 replicates. The  $\Delta$ SSLs values of simulated data were collected and used to estimate their stepwise empirical cumulative density function (cdf), with linear interpolation<sup>4</sup>. The cumulative probability of the observed  $\Delta$ SSLs under the null distribution was calculated, and we defined  $P$ , the significance of an observed site  $\Delta$ SSLs comparison of the species topology  $H_0$ , and an alternative topology  $H_A$  as  $P = 1 - \text{cdf}(\Delta\text{SSLs}_{H_A-H_0})$ . The substitution model for each locus was determined by ProtTest 3.4<sup>57</sup>.

To gauge robustness of the results, we implemented WAG + GAMMA models of amino acid substitution instead of the JTT + GAMMA model in the model fit of sequence convergence. The substitution model did not have significant impact on the convergent genes in the present study. In the comparison of alternative models (convergent tree) and null model (species tree), we introduced 9 trees representing different topologies, but all supported the monophyly of marine mammals (Supplementary Fig. S7). The data presented in this study include genes identified from any pair of hypothesis tests between alternative tree and species tree (with defined  $P$  less than 0.05). We also used the average  $\Delta$ SSLs values from 9 hypothesis tests to detect the correlation between likelihood convergence and adaptive selection.

**Sitewise selection pressure and clade model C.** To estimate the sitewise  $d_N/d_S$  ( $\omega$ ), the codon models M7 (the null model, with  $F3 \times 4$  frequencies) and M8 (sitewise selection)<sup>58</sup> were fitted to each gene using *codeml* in PAML package<sup>54</sup>. We then considered the sitewise  $d_N/d_S$  estimate only from loci where the M8 model was favored. Subsequently, we fitted the clade-specific Clade Model C (and null model M1a)<sup>59,60</sup> and estimated  $d_N/d_S$  on the hypothesized clade of marine mammals. In this model, three separate  $d_N/d_S$  ratios were estimated in marine mammals; 'category 0', denoted  $\omega_0$ , for sites under purifying selection where  $0 < \omega < 1$ ; 'category 1', denoted  $\omega_1$ , for sites evolving neutrally, where  $\omega = 1$ ; and 'category 2', denoted  $\omega_2$ , for sites under diversifying selection, in which  $\omega$  is free and can be larger than 1. In this model, the three  $\omega$  ratios ( $\omega_0$ ,  $\omega_1$  and  $\omega_2$ ) were calculated by ML and the Bayes empirical Bayes (BEB) posterior probabilities and we treated sites under purifying selection, neutrally, diversifying (positive) selection with a BEB posterior for each category  $> 0.5$ .

Gene enrichment analyses were performed using DAVID (Database for Annotation, Visualization and Integrated Discovery)<sup>61</sup> and R Spider<sup>62</sup>. All gene groups are potentially informative despite lower

rankings and serve to guide biological interpretation<sup>63</sup>. Enriched Biological Process (BP) and Molecular function GO terms can be found in Supplementary Table S5 and KEGG pathway analysis can be found in Supplementary Table S6.

## References

1. Zakon, H. H. Convergent evolution on the molecular level. *Brain Behav. Evol.* **59**, 250–261 (2002).
2. Stern, D. L. The genetic causes of convergent evolution. *Nature Rev. Genet.* **14**, 751–764 (2013).
3. Wichman, H. A., Badgett, M. R., Scott, L. A., Boulianne, C. M. & Bull, J. J. Different trajectories of parallel evolution during viral adaptation. *Science* **285**, 422–424 (1999).
4. Parker, J. *et al.* Genome-wide signatures of convergent evolution in echolocating mammals. *Nature* **502**, 228–231 (2013).
5. Woods, R., Schneider, D., Winkworth, C. L., Riley, M. A. & Lenski, R. E. Tests of parallel molecular evolution in a long-term experiment with *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **103**, 9107–9112 (2006).
6. Zhang, J. Parallel adaptive origins of digestive RNases in Asian and African leaf monkeys. *Nat. Genet.* **38**, 819–823 (2006).
7. Berta, A., Sumich, J. L. & Kovacs, K. M. *Marine Mammals, Evolutionary Biology*. Academic Press, San Diego, USA (2006).
8. Hoelzel, A. R. *Marine mammal biology, an evolutionary approach*. Oxford, Blackwell Publishing, Chicago, USA (2002).
9. Wang, Z. *et al.* Adaptive evolution of 5'HoxD genes in the origin and diversification of the cetacean flipper. *Mol Biol Evol.* **26**, 613–622 (2009).
10. Mirceta, S. *et al.* Evolution of mammalian diving capacity traced by myoglobin net surface charge. *Science*. **340**, 1234192 (2013).
11. Foote, A. D. *et al.* Convergent evolution of the genomes of marine mammals. *Nat. Genet.* **47**, 272–275 (2015).
12. Chen, Z. *et al.* Slow skeletal muscle myosin-binding protein-C (MyBPC1) mediates recruitment of muscle-type creatine kinase (CK) to myosin. *Biochem. J.* **436**, 437–445 (2011).
13. Squire, J. M., Luther, P. K. & Knupp, C. Structural evidence for the interaction of C-protein (MyBP-C) with actin and sequence identification of a possible actin-binding domain. *J. Mol. Biol.* **331**, 713–724 (2003).
14. Ottuschtsch, N., Raes, A., Van Hoorick, D. & Snyders, D. J. Obligatory heterotetramerization of three previously uncharacterized Kv channel alpha-subunits identified in the human genome. *Proc. Nat. Acad. Sci. USA* **99**, 7986–7991 (2002).
15. Britton, C. *et al.* Human liver mitochondrial carnitine palmitoyltransferase I, characterization of its cDNA and chromosomal localization and partial analysis of the gene. *Proc. Nat. Acad. Sci. USA* **92**, 1984–1988 (1995).
16. Ramoz, N. *et al.* Mutations in two adjacent novel genes are associated with epidermodyplasia verruciformis. *Nat. Genet.* **32**, 579–581 (2002).
17. Tsai, T. C., Lee, Y. L., Hsiao, W. C., Tsao, Y. P. & Chen, S. L. NRIP, a novel nuclear receptor interaction protein, enhances the transcriptional activity of nuclear receptors. *J. Biol. Chem.* **280**, 20000–20009 (2005).
18. Sinnesael, M. *et al.* Androgen receptor (AR) in osteocytes is important for the maintenance of male skeletal integrity, evidence from targeted AR disruption in mouse osteocytes. *J. Bone Miner Res.* **27**, 2535–2543 (2012).
19. Gao, W. *et al.* Wdr18 is required for Kupffer's vesicle formation and regulation of body asymmetry in zebrafish. *PLoS One*. **6**, e23386 (2011).
20. Gurnett, C. A. *et al.* Myosin binding protein C1, a novel gene for autosomal dominant distal arthrogryposis type 1. *Hum Mol. Genet.* **19**, 1165–1173 (2010).
21. Gupta, M. K. & Robbins, J. Post-translational control of cardiac hemodynamics through myosin binding protein C. *Pflugers Arch.* **466**, 231–236 (2014).
22. Bonnefont, J. P. *et al.* Molecular analysis of carnitine palmitoyltransferase II deficiency with hepatocardiomyopathy expression. *Am. J. Hum. Genet.* **58**, 971–978 (1996).
23. Ivanova, Z. *et al.* Effect of fish and krill oil supplementation on glucose tolerance in rabbits with experimentally induced obesity. *Eur. J. Nutr.* doi: 10.1007/s00394-014-0782-0 (2014).
24. Yang, Z. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. *Mol. Biol. Evol.* **15**, 568–573 (1998).
25. Liu, Y. *et al.* Convergent sequence evolution between echolocating bats and dolphins. *Curr. Biol.* **20**, R53–R54 (2010).
26. Zhou, X. *et al.* Phylogenomic analysis resolves the interordinal relationships and rapid diversification of the laurasiatherian mammals. *Syst. Biol.* **61**, 150–164 (2012).
27. Meredith, R. W. *et al.* Impacts of the Cretaceous Terrestrial Revolution and KPg extinction on mammal diversification. *Science* **334**, 521–524 (2011).
28. Murphy, W. J. *et al.* Molecular phylogenetics and the origins of placental mammals. *Nature* **409**, 614–618 (2011).
29. Murphy, W. J. *et al.* Resolution of the early placental mammal radiation using Bayesian phylogenetics. *Science* **294**, 2348–2351 (2011).
30. Ashkenazy, H. *et al.* FastML, a web server for probabilistic reconstruction of ancestral sequences. *Nucleic Acids. Res.* **40**, W580–W584 (2012).
31. Zhang, J., Nielsen, R. & Yang, Z. Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. *Mol. Biol. Evol.* **22**, 2472–2479 (2005).
32. Zakon, H. H., Lu, Y., Zwickl, D. J. & Hillis, D. M. Sodium channel genes and the evolution of diversity in communication signals of electric fishes, convergent molecular evolution. *Proc. Natl. Acad. Sci. USA* **103**, 3675–3680 (2006).
33. Schiaffino, S. & Reggiani, C. Fiber types in mammalian skeletal muscles. *Physiol. Rev.* **91**, 1447–1531 (2011).
34. Barton, P. J. R. & Buckingham, M. E. The myosin alkali light chain proteins and their genes. *Biochem. J.* **231**, 249–261 (1985).
35. Puitsyn, A., Schlater, A. & Kanatous, S. Transformation of metabolism with age and lifestyle in Antarctic seals, a case study of systems biology approach to cross-species microarray experiment. *BMC Syst. Biol.* **4**, 133 (2010).
36. Gorospe, M., Tominaga, K., Wu, X., Fähring, M. & Ivan, M. Post-Transcriptional Control of the Hypoxic Response by RNA-Binding Proteins and MicroRNAs. *Front Mol Neurosci.* **4**, 7 (2011).
37. Grosset, C. *et al.* A mechanism for translationally coupled mRNA turnover: interaction between the poly(A) tail and a c-fos RNA coding determinant via a protein complex. *Cell* **103**, 29–40 (2000).
38. Anderson, P. & Kedersha, N. Stress granules: the Tao of RNA triage. *Trends Biochem. Sci.* **33**, 141–150 (2008).
39. Somasekharan, S. P. *et al.* YB-1 regulates stress granule formation and tumor progression by translationally activating G3BP1. *J Cell Biol.* **208**, 913–929 (2015).
40. Bollback, J. P. & Huelsenbeck, J. P. Parallel genetic evolution within and between bacteriophage species of varying degrees of divergence. *Genetics* **181**, 225–234 (2009).
41. Wong, A., Rodrigue, N. & Kassen, R. Genomics of adaptation during experimental evolution of the opportunistic pathogen *Pseudomonas aeruginosa*. *PLoS Genet.* **8**, e1002928 (2012).
42. Breidenstein, E. B., Khaira, B. K., Wiegand, I., Overhage, J. & Hancock, R. E. Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. *Antimicrob Agents Chemother* **52**, 4486–4491 (2008).
43. Zou, Z. & Zhang, J. No genome-wide protein sequence convergence for echolocation. *Mol. Biol. Evol.* **32**, 1237–1241 (2015).

44. Thomas, G. W. & Hahn, M. W. Determining the Null Model for Detecting Adaptive Convergence from Genomic Data, A Case Study using Echolocating Mammals. *Mol. Biol. Evol.* **32**, 1232–1236 (2015).
45. Dettman, J. R. *et al.* Evolutionary insight from whole-genome sequencing of experimentally evolved microbes. *Mol. Ecol.* **21**, 2058–2077 (2012).
46. Doolittle, R. Convergent evolution, the need to be explicit. *Trends Biochem. Sci.* **19**, 15–18 (1994).
47. Gallant, J. R. *et al.* Nonhuman genetics. Genomic basis for the convergent evolution of electric organs. *Science* **344**, 1522–1525 (2014).
48. Chan, Y. F. *et al.* Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a Pitx1 enhancer. *Science* **327**, 302–305 (2010).
49. Miller, W. *et al.* 28-way vertebrate alignment and conservation track in the UCSC Genome Browser. *Genome Res.* **17**, 1797–1808 (2007).
50. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
51. Edgar, R. C. Muscle, multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797 (2004).
52. Abascal, F., Zardoya, R. & Telford, M. J. TranslatorX, multiple alignment of nucleotide sequences guided by amino acid translations. *Nucleic Acids Res.* **38**, 7–13 (2010).
53. Suyama, M., Torrents, D. & Bork, P. PAL2NAL, robust conversion of protein sequence alignments into the corresponding codon alignments. *Nucleic Acids Res.* **34**, W609–W612 (2006).
54. Yang, Z. H. PAML 4, phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* **24**, 1586–1591 (2007).
55. Stamatakis, A. RAxML-VI-HPC, maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**, 2688–2690 (2006).
56. Rambaut, A. & Grassly, N. C. Seq-Gen, an application for the Monte Carlo simulation of DNA sequence evolution along phylogenetic trees. *Comput. Appl. Biosci.* **13**, 235–238 (1997).
57. Darriba, D., Taboada, G. L., Doallo, R. & Posada, D. ProtTest 3, fast selection of best-fit models of protein evolution. *Bioinformatics* **27**, 1164–1165 (2011).
58. Yang, Z., Nielsen, R., Goldman, N. & Pedersen, A. M. Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics* **155**, 431–449 (2000).
59. Bielawski, J. P. & Yang, Z. H. A maximum likelihood method for detecting functional divergence at individual codon sites, with application to gene family evolution. *J. Mol. Evol.* **59**, 121–132 (2004).
60. Wong, W. S., Yang, Z., Goldman, N. & Nielsen, R. Accuracy and power of statistical methods for detecting adaptive evolution in protein coding sequences and for identifying positively selected sites. *Genetics* **168**, 1041–1051 (2004).
61. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Bioinformatics enrichment tools, paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **37**, 1–13 (2009).
62. Antonov, A. V., Schmidt, E. E., Dietmann, S., Krestyaninova, M. & Hermjakob, H. R spider: a network-based analysis of gene lists by combining signaling and metabolic pathways from Reactome and KEGG databases. *Nucleic Acids Res.* **38**, 78–83 (2010).
63. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57 (2009).

## Acknowledgements

This work was supported by NIH AG047745 (to V.N.G.).

## Author Contributions

X.Z. and V.N.G. conceived the study. X.Z. performed genetic analyses. X.Z. and V.N.G. wrote the manuscript with significant help from I.S. All authors approved the final version of the manuscript.

## Additional Information

**Supplementary information** accompanies this paper at <http://www.nature.com/srep>

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Zhou, X. *et al.* Convergent evolution of marine mammals is associated with distinct substitutions in common genes. *Sci. Rep.* **5**, 16550; doi: 10.1038/srep16550 (2015).



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>